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Thin-layer chromatography of sugars in plant material

Chromatografia cienkowarstwowa cukrów w materiale roślinnym

Sugars in herbs exist in three forms: monosaccharides, oligosaccharides and polysaccharides. Monosaccharides are also the basic constituent units of oligosaccharides and polysaccharides [14]. Sugars are one of basic, the most common substances in plants. They are spare material, they build the structure of plant cells and they are the main source of energy for the life of plants, animals and humans. They originate in plants from carbon dioxide and water with the use of sun energy. They brake down in oxygen respiration, and thanks to this process the energy for life is gained.

It is difficult to obtain them over from plant material because sugars in cells build the cell wall or, in the form of polysaccharides, they are the main element of cell matrix. Professional literature data [7] divide plant polysaccharides into two kinds: from plant matrix and from plant's cell walls.

The analysis of polysaccharides is difficult for their large molecular weight, complex structures and inert chemical activations [14]. Chromatographic methods, as powerful separation techniques, are extensively applied to the compositional and structural analysis of polysaccharides when combined with other detection methods including UV, IR, MS, NMR. Polysaccharides were analyzed with the use of gas chromatography with MS detection (GC-MS) [16], ion chromatography (IC) [1, 17] and high performance liquid chromatography (HPLC) [12, 15]. Electromigration methods have very high separation efficiency and were also used for the analysis of sugars. In TLC method, polysaccharides were investigated after acid hydrolisis [3, 8]. It is difficult assignment because they need to be derivatized to be seen on a chromatographic plate, they have similar retention times and are difficult to solve in methanol.

Malve arborae, known also as black rose, rose of haven or holy mallow, is a beautiful garden plant. It belongs to *Malvaceae* (L.) family. The plant is found in the Mediteranean Sea region as a wild plant. In many European countries, also in Poland, it is grown as a decorative plant. *Malae arborae flos* is anthocyanin and mucous plant material. It was used in folk medicine and the flower of *Malve arborae* still exists as a medical plant in Polish Pharmacopea 4th edition (Vol. IV).

The main task in this work was to establish the TLC method to detect monosaccharides in the biological material and a practical application of this method to analyze sugars in hydrolysate of polysaccharides gained from *Malve arborae flos* water extract. Experiments with a fraction of cell matrix monosaccharides are the first part of investigations. The TLC analysis of cell walls polysaccharides is also planned.

EXPERIMENTAL DESIGN

SOLVENTS AND STANDARDS

All organic solvents used in the study, of analytical grade, were purchased from POCh (Gliwice, Poland). Standards of sugars were purchased from Merck (Darmstadt, Germany). For the investigations, water-methanol (0.5:9.5, v/v) solutions of 10 sugars and alcohol sugar derivatives were prepared.

Solutions of standards were prepared for analysis with the use of a little amount of water to dissolve monosaccharides. Then methanol was added. Such an operation had to be done because monosacharides are not soluble in methanol.

CHROMATOGRAPHY

Thin layer chromatography (TLC) was conducted on HPTLC silica gel plates 5 x 10 cm, 10 x 10 cm and HPTLC-Diol plates 5 x 10 cm, (Merck, Germany). All chromatographic experiments were carried out in Teflon DS chambers (Chromdes, Lublin, Poland). Chromatograms were obtained in the isocratic technique of development. The distance of development was 8.5 cm. The best mobile phase composition was established in an experimental way (Table 1). Extracts were applied with the use of 10 mL Hamilton syringe on a plate as spots or 3-4 mm zones. 10 μL of standards and 0.5 mL of extract were applied. Elution was performed with the use of mobile phase composed of: 1-propanol, water and ethyl acetate (4:0.5:0.5 v/v/v) for HPTLC silica gel layer and of: 1-propanol and ethyl acetate (3:1 v/v) for HPLC-Diol layer.

Documentation in the form of photographs (Fig. 1, 2) was done with Olympus FE-150 photocamera.

Table 1. List of tested TLC sets in monosaccharides analysis

No.	Stationary phase	Eluent composition	References
1	chromatography paper	1-butanol:piridine: water (6:4:3 v/v/v)	8
2		ethyl acetate : 99.5% acetic acid : 80% formic acid : water (18:3:1:4 v/v/v/v)	
3	Silica gel 60	acetone : water (15 : 85 v/v)	10
4	peri-celulose	ethyl acetate : piridine : 99.5% acetic acid : water (5:5:3:1 v/v/v/v)	17
5	Silica gel 60	1-propanol : ethyl acetate : water (7:2:1 v/v/v)	14, own experiments
6		dichlorometane : methanol : 99.5% acetic acid : water (50:50:25:10 v/v/v/v)	
7		2-propanol : 0.75% water solution of boric acid : 99.5% acetic acid (40:5:1 v/v/v)	
8		acetonitrile : water : 98 % hydrochloric acid (17:3:0.1 v/v/v)	
9		1-butanol : 1-propanol : acetic acid : water (30:10:10:10 v/v/v/v)	
10		acetonitrile : water (7:3 v/v)	
11		1-propanol : ethyl acetate : water (4:0.5:0.5 v/v/v)	
12	HPTLC-Diol	1-propanol : ethyl acetate : 80% formic acid (3: 1:0.3 v/v/v)	own experiments
13		1-propanol : ethyl acetate (3:1 v/v)	
14		1-propanol : ethyl acetate : 80% formic acid (2: 1: 0.1 v/v/v)	
15		1-propanol : ethyl acetate : water (3:1:0.3 v/v/v)	

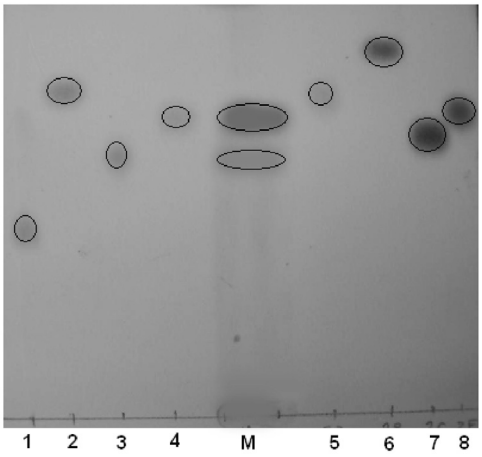

Fig. 1. Identification of galactose and fructose in hydrolysate of holly mallow polysaccharides; Si 60 HPTLC / 1-propanol : ethyl acetate : water (4:0.5:0.5 v/v/v). 1 – lactose, 2 – sorbose 3 – galactose, 4 – glucose, 5 – arabinose, 6 – xylose, 7 – saccharose, 8 – fructose, M – hydrolysate of polisaccharides

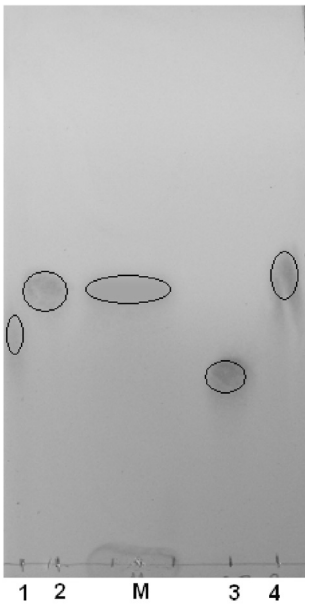

Fig. 2. Identification of glucose in hydrolysate of holly mallow polysaccharides; HPTLC – Diol / 1-propanol : ethyl acetate (3:1 v/v). 1 – galactose, 2 – glucose, 3 – saccharose, 4 – fructose, M – hydrolysate of polisaccharides

PLANT MATERIAL

The material for the investigations were dried and crushed flowers of holly mallow (*Malve arborae flos*). The method of isolation and hydrolysis of water soluble polysaccharides fraction was based on literature data [3].

To isolate polysaccharides, water extract was prepared (5 g plant material and 100 mL destilated water). The extraction process was conducted in ultrasonic bath at the temperature of 75° C for one hour [8]. The extract was filtrated and 210 mL of 95% ethanol was added. This mixture was left at the temperature of 4° C for 12 hours [10]. The sediment of polysaccharides was filtrated and hydrolyzed with the use of 15 mL of 5 % water solution of sulphuric acid (VI) for two hours at 90° C. When the solution was cold, the neutralization process was conducted with the use of solid $BaCO_3$. Precipitation of $BaSO_4$ was the filtrated and filtrate was used for TLC experiments [5].

DETECTION

Different reagents were examined to visualize standards of sugars. Results of the experiments with different solutions are listed in Table 2. For further experiments reagent number IV was chosen, because it can be stored for 14 days and is relatively easy to prepare. It is a metanolic solution of diphenylamine, aniline and orthophosphoric (V) acid [6].

Table 2. List of tested sugar derivation reagents

Number of reagent	Composition of solution to derivation	Preparation of the reagent
I	solution of kalium hypermanganicum (VII) in sodium base [13], chromatogram heated after sprayng at 100 °C	Dissolve 0.5 g $KMnO_4$ in 100 mL 0.1 mol/L NaOH
II	10 % (v/v) methanolic solution of sulfuric acid (VI), chromatogram heated after spraying at 100 °C till spots were seen	Dissolve 10mL of 95% of sulphuric acid (VI) in 82 mL of methanol
III	1-butanolic solution of orthophosphoric acid (V) and aniline [14], chromatogram heated after spraying in 130 °C till spots were seen	Prepare two 1-butanolic solutions of 15% aniline (I) and 30% orthophosphoric acid (II). Mix them just before use (20 mL of (I) and 50 mL of (II)), till dissolving of sediment.
IV	metanolic solution of diphenylamine, aniline and orthophosphoric acid (V) [14], chromatogram heated after spraying at 150 °C for 4 minutes	Dissolve 2 g of diphenylamine and 2 mL of aniline in 80 mL of methanol. Add 15 mL of 30 % ortophosphoric acid (V) and fill up to 100 mL with methanol
V	etanolic solution of anishaldehyde and 95 % sulphuric acid (VI) [15], chromatogram heated after spraying till spots were seen	Mix just before use 9 mL of ethanol, 0.5 mL of 95 % sulphuric acid (VI) and 0.5 mL of anishaldehyde
VI	mixture of etanolic solution of α-naphtol, 95 % sulphuric acid (VI), ethanol and water [16], chromatogram heated after spraying at 100 °C till spots were seen	Mix 10.5 mL 15% etanolic solution of α-naphtol, 6.5 mL of 95 % sulphuric acid (VI), 40.5 mL of 96% ethanol and 4 mL of water

RESULTS AND DISCUSSION

Sugars are very important, the most common substances in plant material but they are also very difficult to analyze in TLC technique. The first step in sugar analysis was to establish the best method of derivation standards of sugars on chromatogram. Six methods of detection were tested (Figs. 3–9).

$KMnO_4$ reagent was applied as reducting sugars indicator (Fig. 3). With the use of H_2SO_4 reagent the reaction with organic substances was checked (Fig. 4). It was not specific for sugars. The specific mechanism of sugars visualization is shown in Figure 5. This group of methods is based on the reaction of the cyclic form of sugar derivative with phenyl group in acid medium. The color of the product is often characteristic of one kind of sugar. Results are shown in Figures 6–9.

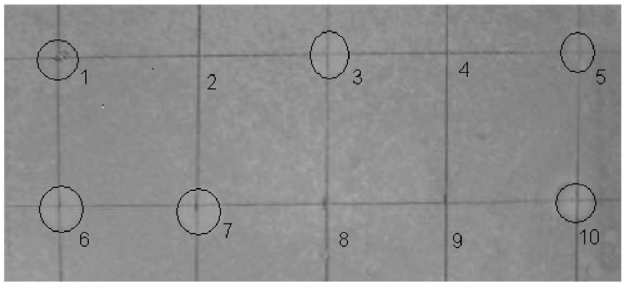

Fig. 3. Effect of derivation with the use of reagent no. I – sorbit (1), lactose (2), sorbose (3), galactose (4), glucose (5), arabinose (6), xylose (7), saccharose (8), mannit (9), fructose (10)

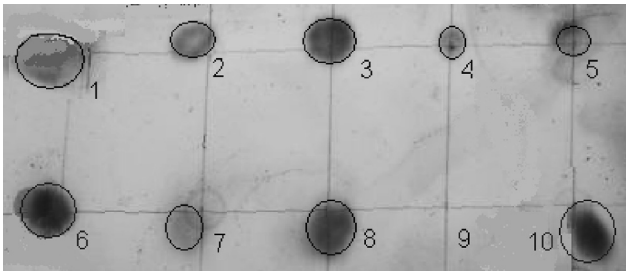

Fig. 4. Effect of derivation with the use of reagent no. II – sorbit (1), lactose (2), sorbose (3), galactose (4), glucose (5), arabinose (6), xylose (7), saccharose (8), mannit (9), fructose (10)

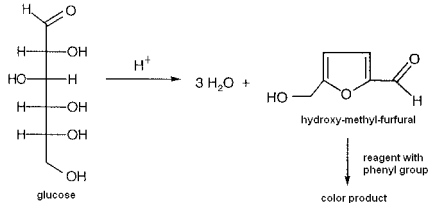

Fig. 5. Scheme of getting the color product during sugars derivation [13]

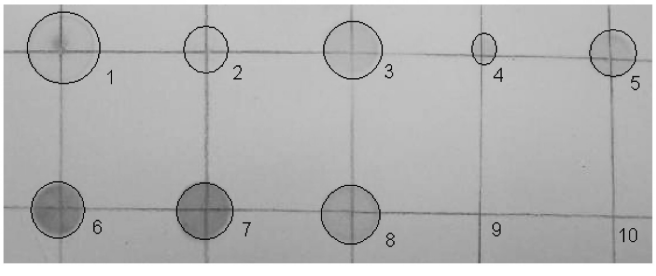

Fig. 6. Effect of derivation with the use of reagent no. III – sorbit (1), lactose (2), sorbose (3), galactose (4), glucose (5), arabinose (6), xylose (7), saccharose (8), mannit (9), fructose (10)

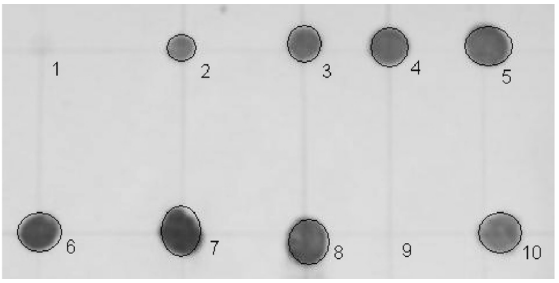

Fig. 7. Effect of derivation with the use of reagent no. IV – sorbit (1), lactose (2), sorbose (3), galactose (4), glucose (5), arabinose (6), xylose (7), saccharose (8), mannit (9), fructose (10).

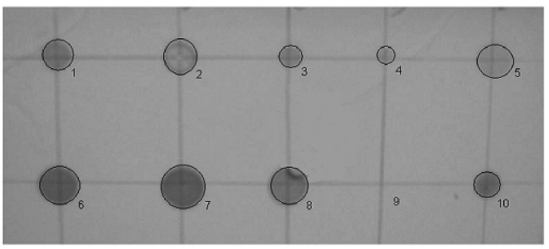

Fig. 8. Effect of derivation with the use of reagent no. V – sorbit (1), lactose (2), sorbose (3), galactose (4), glucose (5), arabinose (6), xylose (7), saccharose (8), mannit (9), fructose (10)

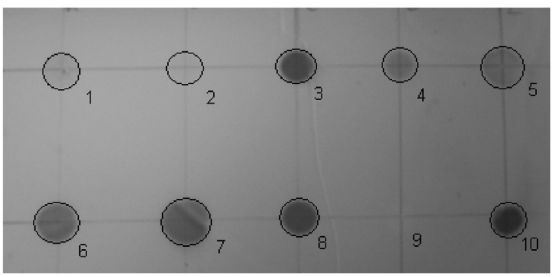

Fig. 9. Effect of derivation with the use of reagent no. VI – sorbit (1), lactose (2), sorbose (3), galactose (4), glucose (5), arabinose (6), xylose (7), saccharose (8), mannit (9), fructose (10)

Monosaccharides of one polysaccharides fraction in *Malve arborae flos* were investigated with the use of planar chromatography after acidic hydrolysis of cell matrix polysaccharides. Tests with several mobile phases were done. The best composition of eluents for both stationary phases was set experimentally.

At silica stationary phase, the best standard separation and identification in one fraction of polysaccharides from *Malvae arborae flos* was achieved with the use of a mixture of polar solvents with water. The addition of water assured good shape of spots. The mobile phase composition was modified from literature data [6]. In hydrolysate of *Malve arborae* polysaccharides galactose (R_f 0.6) was identified (Fig. 1). The presence of lactose (R_f 0.43), sorbose (R_f 0.73), arabinose (R_f 0.71), saccharose (R_f 0.55) and xylose (R_f 0.79) was counted out. Fructose (R_f 0.67) and glucose (R_f 0.67) separation was not achieved in this chromatographic set.

Investigations of monosaccharides separation with the use of HPTLC-Diol stationary phase were done for the first time. For the experiments, a group of four sugars was selected: galactose (R_f 0.39), glucose (R_f 0.46), saccharose (R_f 0.33) and fructose (R_f 0.49). It was settled that addition of water to mobile phases worsened the separation and shape of spots. The best separation of sugars on HPTLC-Diol plates was achieved with a mixture of 1-propanol and ethyl acetate as a mobile phase (Fig. 2). In this chromatographic set glucose was identified.

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SUMMARY

In the present publication experiments were concentrated on elaborating a simple method of separation standards of monosaccharides and choosing the best practically applied method of derivation. Sugars and their alcohol derivatives were analyzed. Six methods of monosaccharides detection were tested. Two stationary phases with several eluents were investigated. The method was used to analyze hydrolyzed monosaccharides from *Malvae arborae flos* polysaccharides. The samples were hydrolyzed into monosaccharide units with an acidic reagent – sulfuric acid (VI). On silica stationary phase, galactose was identified in hydrolysate of one fraction of *Malve arborae* polysaccharides. Investigations of monosaccharides separation with the use of HPTLC-Diol stationary phases was conducted for the first time. The best separation was achieved with a mixture of 1-propanol and ethyl acetate as a mobile phase. In this chromatographic set glucose was identified.

STRESZCZENIE

W pracy przedstawiono prostą metodę rozdziału wzorców monosacharydów i ich pochodnych alkoholowych oraz szereg (sześć) metod derywatyzacji cukrów prostych. Wykorzystano dwie fazy stacjonarne (żel krzemionkowy oraz żel modyfikowany grupami diolowymi) oraz kilkanaście faz ruchomych. Metodę wykorzystano następnie do analizy hydrolizatu polisacharydów z kwiatów malwy czarnej (*Malvae arborae flos*). Do hydrolizy próbek został użyty kwas siarkowy (VI). Na żelu krzemionkowym zidentyfikowano galaktozę. Płytki modyfikowane grupami diolowymi zostały wykorzystane do analizy monosacharydów po raz pierwszy. Najlepszy rozdział osiągnięto stosując mieszaninę 1-propanolu i octanu etylu jako fazę ruchomą. W tym układzie chromatograficznym zidentyfikowano glukozę.